Decoration of chondroitin polysaccharide with threonine: synthesis, conformational study and ice-recrystallization inhibition activity

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Abstract

Several threonine (Thr)- and/or alanine (Ala)-rich antifreeze glycoproteins (AFGPs) and polysaccharides act in nature as ice recrystallization inhibitors. Among them, the Thr-decorated capsular polysaccharide (CPS) from the cold-adapted *Colwellia psychrerythraea* 34H bacterium was recently investigated for its cryoprotectant activity. A semi-synthetic mimic thereof was here prepared from microbial sourced chondroitin through a four step strategy, involving a partial protection of the chondroitin polysaccharide as a key step for gaining an unprecedented quantitative amidation of its glucuronic acid units. In-depth NMR and computational analysis suggested a fairly linear conformation for the semi-synthetic polysaccharide, for which the antifreeze activity by a quantitative ice recrystallization inhibition assay was measured. We compared the structure-activity relationships for the Thr-derivatized chondroitin and the natural Thr-decorated CPS from *C. psychrerythraea*.

Keywords: chondroitin, semi-synthesis, threonine, antifreeze, conformational study

Introduction

Glycosaminoglycans (GAGs) are polysaccharides ubiquitously distributed in the animal kingdom, playing several biological functions. From a structural point of view, they often consist of a linear backbone of variously sulfated

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disaccharide repeating units, with alternating uronic acid and *N*-acetyl-hexosamine residues. One of the most widespread GAGs is chondroitin sulfate (CS), that is widely distributed in mammals and invertebrates, as well as in some bacteria. It is a polysaccharide with a molecular weight usually slightly lower than 50 kDa, consisting of glucuronic acid (GlcA) and *N*-acetyl-galactosamine (GalNAc) units linked together through alternating β-1→3 and β-1→4 glycosidic bonds and decorated with sulfate groups to a various extent (Figure 1a).[1] Unsulfated variants of CS have been also found in nature. They are produced by some bacteria as exopolysaccharides.[2,3]

CS is commonly used for the therapy of tibiofibular osteoarthritis of the knee. It is also employed for the treatment of burns and as a component of viscoelastic solutions used as surgical aids. Many other pharmacological and biomedical applications are currently under development,[4] with a particular focus on the field of drug delivery and tissue engineering. In the last two decades many works appearing in the literature have reported chemical and chemo-enzymatic derivatization of CS or unsulfated chondroitin, aiming to access modified polysaccharide structure with tailored biomedical applications.[5-10] Among the derivatization reactions, carboxyl group modification with amines or hydrazides is very common. Indeed, CS polysaccharide has been coupled through amide linkages with several small biomolecules – tyramine,[11,12] histamine,[13] biotin,[14] cholesterol[15] among others – as well as biomacromolecules, such as tissue proteins and hyaluronic acid.[16-19] Nonetheless, to the best of our knowledge no systematic study of how the activation conditions of the carboxylic acid of chondroitin or CS influence the degree of substitution (DS) has been done yet. Furthermore, diverse DS for amidation of such polysaccharides has been obtained, with values ranging from 0.01 to 0.88.[10]

Colwellia psychrerythraea 34H is a strictly psychrophilic Gram-negative bacterium isolated in Arctic marine sediments.[20] It has been reported that bacterial adaptation to low temperatures can be driven by several different mechanisms, including the production of antifreeze (glyco)proteins and small molecules as cryoprotectants.[21] Very recently, it has been found that *C. psychrerythraea* 34H uses an alternative, unprecedented mechanism, producing polysaccharides with ice recrystallization inhibition (IRI) activity.[22,23] One of them, a capsular polysaccharide (CPS), possesses a linear tetrasaccharide repeating unit with alternating *N*-acetyl-hexosamines – GalNAc and *N*-acetyl-glucosamine (GlcNAc) – and uronic acids [GlcA and galacturonic acid (GalA)] in a GAG-like fashion. A threonine (Thr) amide decoration on carboxylic acid of GalA units complete the structure (Figure 1b). It was suggested to confer the cryoprotectant activity to the CPS,[22] in resemblance with typical Thr-rich antifreeze (glyco)proteins.[24]

Since antifreeze molecules can find applications as cell, tissue, organ and frozen food cryopreservatives as well as cryosurgical aids, the design of ice recrystallization inhibitors that mimic the structure of natural products with antifreeze activity is an emerging field in synthetic organic and polymer chemistry. To this aim, both small molecules and macromolecules were obtained through total synthetic approaches and tested for antifreeze activity.[25-27] The GAG-like structure of the CPS from *C. psychrerythraea* 34H suggested us to obtain an unprecedented chondroitin polysaccharide

with a Thr amide decoration on GlcA units. Actually, unsulfated chondroitin polysaccharide has been demonstrated to have no IRI activity.[22] Therefore, it would be interesting to test whether a decoration of the chondroitin backbone with Thr amide moieties is able to elicit IRI activity or not. A semi-synthetic approach could be employed for the production of Thr-decorated chondroitin, taking advantage of the high availability of pure unsulfated chondroitin from the fed-batch fermentation of *Escherichia coli* K4 followed by a suitable downstream purification.[28] This polysaccharide has been previously employed as starting material for the obtainment of non-animal sourced CS and fucosylated CS polysaccharides through tailored semi-synthetic strategies.[29-33] Here a study of chondroitin carboxylic acid activation conditions was done in order to achieve the highest possible DS of Thr decoration of the polysaccharide backbone, then followed by evaluation of the 3D-structure and IRI activity of the obtained semi-synthetic polymer.

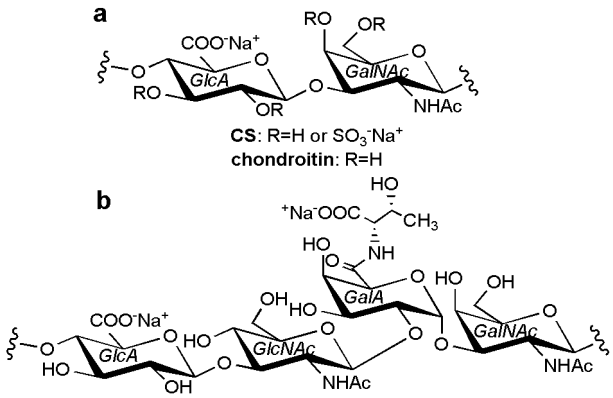


Figure 1: Repeating units of (a) CS and unsulfated chondroitin, and (b) CPS from *C. psychrerythraea* 34H

Experimental

General methods

Commercial grade reagents and solvents were used without further purification, except where else specified. The term "pure water" refers to water purified by a Millipore Milli-Q Gradient system (≥18.2 Ω mean resistivity). Phosphate-buffered saline (PBS) solution was prepared using preformulated tablets in 200 mL of pure water to give [NaCl] = 0.138 M, [KCl] = 0.0027 M, and pH 7.4. Centrifugations were performed with an Eppendorf Centrifuge 5804R instrument at 4°C (4600 g, 10 min). Dialyses were conducted on Spectra/Por 3.5 kDa cut-off membranes at 4°C. Size-exclusion chromatographies were performed on a Bio-Gel P2 column (0.75 x 67.5 cm, Bio-Rad) using 50 mM ammonium bicarbonate as a buffer at a flow rate of 0.2 mL/min. The column eluates were monitored continuously with a Knauer K-2310 refractive index refractometer. Lyophilisation was performed with a 5Pascal Lio 5P 4K freeze dryer. NMR spectra were recorded on a Bruker DRX-600 (^{1}H: 600 MHz, ^{13}C: 150 MHz) instrument equipped with a cryo probe, in D_2O (acetone as internal standard, ^{1}H: $(CH_3)_2CO$ at δ 2.22 ppm; ^{13}C:$(CH_3)_2CO$ at δ 31.5 ppm) or DMSO-d_6 (^{1}H: CHD_2SOCD_3 at δ 2.49 ppm; ^{13}C: CD_3SOCD_3 at δ 39.5 ppm). Gradient-selected COSY, phase sensitive NOESY, and TOCSY

experiments were performed using spectral widths of 6000 Hz in both dimensions, using data sets of 4096 × 256 points. Quadrature indirect dimensions were achieved through States-TPPI method; spectra were processed applying an unshifted Qsine function to both dimensions and data matrix was zero-filled by factor of 2 before Fourier transformation. The mixing time for TOCSY experiment was 120 ms. NOESY experiments were performed at mixing times of 50, 70, 100, 150, and 200 ms, in order to identify genuine NOEs effects. A NOESY experiment with mixing time of 150 ms was performed in H_2O/D_2O 9:1 too. HSQC-DEPT experiments were measured in the ^1H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048 × 256 points and typically 32 increments.

*Preparation of polysaccharide **2a***

Chondroitin sodium salt (22.0 mg, 54.9 μmol) – obtained from fed-batch fermentation of *Escherichia coli* K4 followed by downstream purification steps as already described in literature[28] – was dissolved in pure water (1.0 mL) and passed through a short Dowex 50 WX8 column (H^+ form, 20-50 mesh, approx. 5 cm^3). Elution with pure water was continued until pH of the eluate was neutral. Freeze-drying of the collected eluate gave chondroitin (20.0 mg, 52.8 μmol), that was suspended in pure water (4.0 mL) and treated with EDC (22.7 mg, 0.146 mmol) and NHS (8.5 mg, 73.9 μmol). After 40 min stirring at rt, MES (40.0 mg, 0.204 mmol; pH 6) and then Thr methyl ester **1** (35.7 mg, 0.264 mmol) were added and stirring was continued overnight. Few drops of 1M NaOH solution were then added to adjust pH to 12. After 6 hours stirring at rt, the solution was then neutralized with 1M HCl. Dialysis and subsequent freeze-drying afforded polysaccharide **2a** (17.2 mg) as a white waxy solid.

*Preparation of polysaccharides **2b** and **2c***

Chondroitin TBA salt (31.8 mg, 51.3 μmol) – obtained from chondroitin sodium salt according to a known procedure[34] – was suspended in DMF (2.1 mL) that was freshly dried over activated 4Å molecular sieves. The mixture was heated to 80°C and stirred for 3 hours, after that a clear solution was obtained. After cooling to rt, in the case of **2b** the solution was treated with 0.27M solutions of pyBOP® and HOBt in freshly dried DMF (376 μL each, 0.102 mmol), then with a 0.48M solution of **1** in freshly dried DMF (300 μL, 0.144 mmol), and finally with DIPEA (88.8 μL, 0.510 mmol) that was freshly dried over activated 4Å molecular sieves. Alternatively, in the case of **2c**, the solution was treated with a 0.44M solution of TBTU in freshly dried DMF (229 μL, 0.101 mmol), then with a 0.48M solution of **1** in freshly dried DMF (300 μL, 0.144 mmol), and finally with 2,4,6-collidine (66.5 μL, 0.503 mmol) that was freshly dried over activated 4Å molecular sieves. Both solutions were stirred at rt overnight and then treated with few drops of 1M NaOH aqueous solution. After a further 6 hours stirring, the solutions were neutralized by adding a 1M HCl aqueous solution and then dialyzed and freeze-dried. Since the ^1H-NMR spectrum of both products clearly displayed the signals of residual TBA ions, a further

purification was made by dissolving the polysaccharides in pure water (3.0 mL) and then passing the obtained solutions through a short Dowex 50 WX8 column (H^+ form, 20-50 mesh, approx. 5 cm^3). Elution with pure water was continued until pH of the eluates was neutral. The obtained solutions were then treated with 1M NaOH to adjust the pH to 12 and then neutralized by adding 1M HCl. Dialysis and subsequent freeze-drying of the afforded polysaccharides **2b** (22.0 mg) and **2c** (24.2 mg), respectively, as white waxy solids.

*Preparation of polysaccharide **3***

Chondroitin in free acid form (56.3 mg, 0.148 mmol) – obtained from chondroitin sodium salt as indicated above for the preparation of **2a** – was suspended in DMF (2.8 mL) that was freshly dried over activated 4Å molecular sieves. The mixture was heated to 80°C and stirred for 3 hours, after that a clear solution was obtained. After cooling to rt, it was treated with α,α-dimethoxytoluene (222 μL, 1.48 mmol), that was freshly dried over activated 4Å molecular sieves, and then with a 0.21M solution of (+)-camphor-10-sulfonic acid in freshly dried DMF (178 μL, 37.4 μmol). After overnight stirring at 80°C, a yellowish solution was obtained. It was cooled to rt and treated firstly with triethylamine (0.4 mL) and then with diisopropyl ether (10 mL). The mixture was cooled to -30°C for 2 hours. A white flocculant solid was obtained. It was isolated by centrifugation and then desiccated under vacuum overnight to afford **3** (89.0 mg) as a yellowish amorphous solid.

*Preparation of polysaccharide **4***

A suspension of **3** (27.2 mg, 58.2 μmol) in freshly dried DMF (2.2 mL) was heated at 80°C. After two hours stirring, a clear solution was obtained. It was cooled to rt and then treated with a 0.44M solution of TBTU in freshly dried DMF (323 μL, 0.142 mmol), then with a 0.22M solution of **1** in freshly dried DMF (790 μL, 0.174 mmol), and finally with 2,4,6-collidine (93.8 μL, 0.709 mmol), that was freshly dried over activated 4Å molecular sieves. After overnight stirring at rt, the solution was treated with diisopropyl ether (7.0 mL). A white precipitate was obtained. It was collected by centrifugation and then desiccated under vacuum overnight to give **4** (34.8 mg).

*Preparation of polysaccharide **2d***

Derivative **4** (29.9 mg) was suspended in ethyl acetate (560 μL) and treated with a 0.27M solution of $NaBrO_3$ in pure water (560 μL, 0.151 mmol), and a 0.24M solution of $Na_2S_2O_4$ in pure water (535 μL, 0.128 mmol). The mixture was vigorously stirred at rt overnight under visible light irradiation, and then centrifuged. The supernatant was diluted with water (25 mL) and ethyl acetate (25 mL). The aqueous phase was collected, concentrated, dialyzed and freeze-dried. The obtained white powder (11.0 mg) was dissolved in pure water (5.0 mL) and treated with 1M HCl to adjust to pH 2. The

solution was stirred at 50°C for 1 hour, then 1M NaOH was added to adjust to pH 12. Further stirring was conducted at rt for 6 hours. The solution was then neutralized by adding 1M HCl, and dialyzed. Freeze-drying afforded a white solid that was further purified by filtration through a Sep-pak C-18 cartridge to give pure **2d** (7.5 mg) as a white waxy solid.

Molecular weight determination by Static Light Scattering

SLS measurements were performed with a home-made instrument composed by a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW light source operating at 5325 Å, a photomultiplier (PMT-120-OP/B) and a correlator (Flex02-01D) from Correlator.com. All measurements were performed at (25.00 ± 0.05) °C with temperature controlled through the use of a thermostat bath. Stock solutions of pure chondroitin and of derivatives **2a**, **2b**, **2c**, and **2d** were prepared at 4.9 mg mL^{-1}, 1.05 mg mL^{-1}, 1.12 mg mL^{-1}, 3.4 mg mL^{-1}, and 1.24 mg mL^{-1}, respectively. Deionized water filtered (0.22μm) was used in all cases. The molecular weight of each compound was determined in duplicate. The mass-average molecular weight, M_w, from the below equation was obtained:[35]

$$\frac{k_{ls}c}{R} = \frac{1}{M_w} + 2Bc \quad 1 + \frac{R_g^2}{3}q^2 \tag{1}$$

where c is the sample mass concentration, B the second virial coefficient, $k_{ls} = 4\ ^2 n_o^2 (dn/dc)^2 / \left(N_A\ ^4\right)$ where $n_0 = 1.33$ is the refractive index of water, $dn/dc = 0.185$ is the refractive index increment,[36] N_A is the Avogadro's number, λ is the laser wavelength in vacuum, R_θ is the excess Rayleigh ratio at 90°. The values of R_θ were obtained from $R = \left(I_s \quad I_{s,0}\right) / I_{s,R}\left(n_0^2 / n_R^2\right) R_{,R}$ where I_s is the scattered intensity of the solution, $I_{s,0}$ is the scattered intensity of water, $I_{s,R}$ is the scattering intensity of toluene (the standard) and, $n_R = 1.496$ and $R_{\theta,R} = 2.85\ 10^{-5}$ cm^{-1} are the refractive index and the Rayleigh ratio of toluene respectively,[37] $q = \left(4\ n/\ \right) sin(\ /2)$ and R_g is the radius of gyration.

Conformational study

A simplified model of the Thr-decorated chondroitin polysaccharide **2d** with six repetitions was constructed through the carbohydrate builder within the Glycam web server (Woods Group GLYCAM Web; Complex Carbohydrate Research Center, University of Georgia, Athens, GA, 2005–2014; http://www.glycam.com) while the Thr residue attached to the GlcA unit was constructed employing the builder module in the Maestro package of the Schroedinger Suite 2014. Restrained simulated annealing (SA) calculations were performed on polysaccharide using the AMBER 14.0 package[38] with sugars described by the latest GLYCAM06 force field (GLYCAM_06j-1).[39] Parameters for Thr residue were retrieved from the ff14sb force field within the AMBER 14.0 package as well as missing bond parameters. For annealing

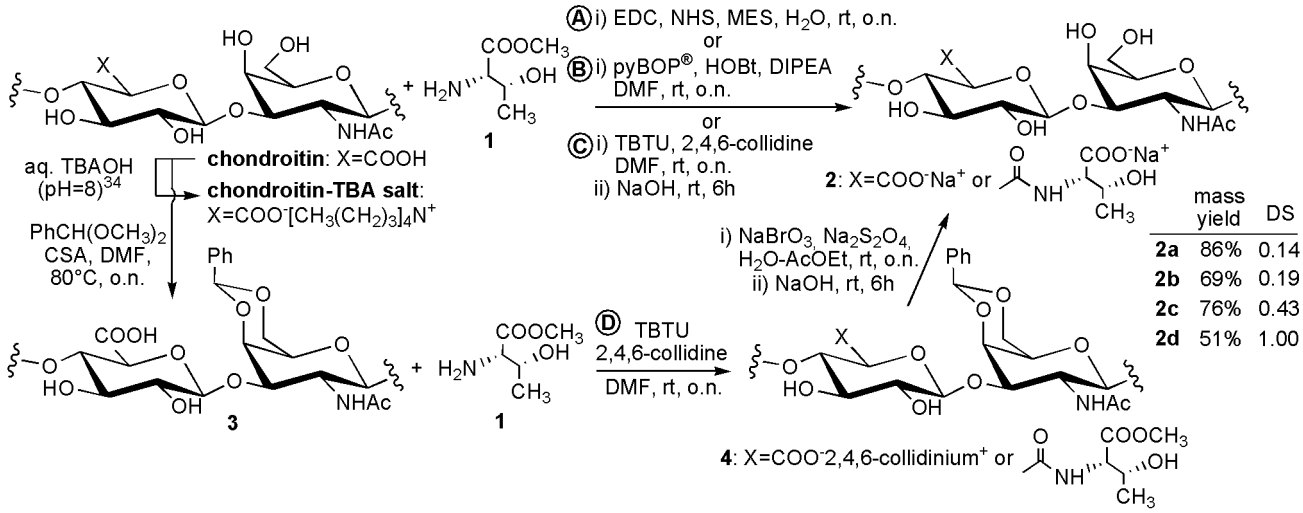
simulations, the General Born solvation (igb = 2) with monovalent salt concentration corresponding to 0.1 M was used. The complex was heated to 600 K in the first 5 ps, cooled to 100 K for the next 13 ps, and then cooled to 0 K for the last 2 ps. The temperature of the system was maintained with a varying time constant: 0.4 ps during heating, 4 ps during cooling to 100 K, 1 ps for the final cooling stage, and then reduced from 0.1–0.05 for the last picosecond. The force constants for NOE constraints were increased from 3 to 30 kcal mol^{-1} $Å^{-2}$ during the first 5 ps and then maintained constant for the rest of the simulation. These force constants were applied in the form of a parabolic, flat-well energy term. The upper distance bounds were retrieved by NOE cross-peak volume integrations performed with the program iNMR (www.inmr.net), using the NOESY experiment collected at mixing time of 100 ms. The NOE volumes were then converted to distance restraints after they were calibrated using the known fixed distance (*H*-6a/*H*-6b of GalNAc). An unrestrained energy minimization step completed the SA run. This SA/energy minimization procedure was repeated 400 times. SA simulations were then analysed by clustering the resulting polysaccharide **2d** conformations through the average linkage method and a cluster member cutoff of 1.5 Å root-mean-squared difference (rmsd) calculated on the sugars rings atoms belonging to the central four dimers. This clustering allowed selecting 38 different conformational clusters for which the most populate one had a frequency of occurrence of 110/400 conformations. Moreover, conformations of this latter cluster feature the lowest overall potential energy and NMR restraint violations. Thus, the representative structure (i.e., the closest to the centroid of the cluster) of this cluster was considered for subsequent molecular dynamics (MD) simulations. After charge neutralization by the addition of 6 Na^+ ions, the complex was solvated with 9381 water molecules in a truncated octahedral box of pre-equilibrated TIP3P water.[40] Several equilibration steps were performed comprising minimization of the solvent molecules with the polysaccharide fixed, minimization of the whole system, and slow heating to 300 K with weak positional restraints on M1 atoms under constant-volume conditions. The following 20 ns production runs were applied in the NPT ensemble. The particle mesh Ewald method[41,42] was used to evaluate the electrostatic interactions with a direct space sum cutoff of 10 Å. With the bond lengths involving hydrogen atoms kept fixed with the SHAKE algorithm, a time step of 2 fs was employed.[43] Related conformational substates populated during the MD simulation were analyzed with the AMBERS's PTRAJ module.[44] To investigate the thermodynamic parameters of the water molecules around the polysaccharide, the GIST program included in cpptraj module in AMBER 14 was used.[45] We set the GIST grid size to 0.75 $Å^3$, and set the GIST analysis region to cover the whole polysaccharide structure. The box sizes were 90 × 90 × 90 $Å^3$. Illustrations of the structures were generated using Chimera.[46]

Ice Recrystallization Inhibition activity

IRI activity was measured using a modified splay assay.[47] A 10 μL sample of polysaccharide or polymer dissolved in PBS buffer (pH 7.4) was dropped onto a chilled glass coverslip set on a piece of polished aluminium placed on dry ice. Upon hitting the chilled glass coverslip, a wafer with diameter of approximately 10 mm and thickness 10 μm was formed instantaneously. The glass coverslip was transferred onto the Linkam cryostage and held at −8 °C under N_2 for 30 minutes. Photographs were obtained using an Olympus CX 41 microscope with a UIS-2 20×/0.45/∞/0–2/FN22 lens and crossed polarizers (Olympus Ltd, Southend on sea, UK), equipped with a Canon DSLR 500D digital camera. Images were taken of the initial wafer (to ensure that a polycrystalline sample had been obtained) and after 30 minutes. Image processing was conducted using Image J, which is freely available. In brief, ten of the largest ice crystals in the field of view were measured and the single largest length in any axis recorded. This was repeated for at least three wafers and the average (mean) value was calculated to find the largest grain dimension along any axis. The average of this value from three individual wafers was calculated to give the mean largest grain size (MLGS). This average value was then compared to that of a PBS buffer negative control providing a way of quantifying the amount of IRI activity. This testing method ensures that positive results are only reported if all ice crystals are inhibited, as opposed to an average per wafer estimation, which would smooth out the presence of rouge ice crystal growth.

Results and Discussion

Several different carboxylic acid activators have been developed for amide bond formation. By limiting to the most common ones, they can be classified according to their structure into phosphonium and uronium/guanidinium salts, carbodiimides, and triazines.[48,49] Amidation of GlcA units of CS was performed in all reported cases with carbodiimide reagents such as water-soluble *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) or, more rarely, dicyclohexylcarbodiimide (DCC), with or without a protic additive such as 1-hydroxybenzotriazole (HOBt) or *N*-hydroxysuccinimide (NHS).[10] Hyaluronic acid – the only unsulfated GAG found in animal tissues – was coupled with amines using not only carbodiimide activators of GlcA carboxylic acid but also in the presence of triazine species. Nonetheless, variable DSs, very often rather far from a quantitative value, were obtained.[50] Our first attempt to decorate unsulfated chondroitin with Thr was made by reaction of the polysaccharide with Thr methyl ester **1**[51] in the presence of EDC and NHS in 4-morpholineethanesulfonic acid (MES) buffered water (pH = 6) (Scheme 1). After overnight reaction at rt, ester cleavage was conducted in one pot by adding sodium hydroxide. Dialysis and subsequent freeze-drying furnished polysaccharide **2a** in 86% mass yield, that was subjected to ^{1}H NMR analysis for evaluation of Thr amide DS. By integration of the CH_3 signal of Thr residue (δ 1.20 ppm) with respect to that of the acetyl (δ 2.01 ppm), a 0.14 DS



Scheme 1: Decoration of chondroitin polysaccharide with Thr amides

value was obtained (Figure 2). In order to enhance DS different carboxylic acid activation conditions were then tested. Since amide couplings in solution with either phosphonium or uronium/guanidium salts are usually performed in DMF, chondroitin was firstly converted into its tetrabutylammonium (TBA) salt according to a known procedure using aqueous TBAOH,[34] in order to enhance its solubility in such solvent. A clear solution of chondroitin-TBA salt in DMF was then treated at rt with a phosphonium salt activator such as (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (pyBOP®) in the presence of HOBt and *N*,*N*-diisopropylethylamine (DIPEA) as a base. Alternatively, the amide coupling was conducted with an uronium salt activator such as *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and 2,4,6-collidine as base. After one-pot cleavage of Thr methyl ester, dialysis, and freeze-drying, integration of ^{1}H-NMR signals allowed DS evaluation of the obtained polysaccharides. In pyBOP®/HOBt case (**2b**, Scheme 1) only a slight increase of DS (0.19) with respect to EDC/NHS reaction was observed, whereas a much significant enhancement was obtained for polysaccharide **2c** (DS = 0.43), nonetheless the coupling reaction was still very far from affording a quantitative Thr decoration.

It was hypothesized that low DS values could be due to a strong network of hydrogen bonds between carboxylic acid and hydroxyl moieties of chondroitin, that should be rather effective especially in an aprotic solvent such as DMF. This could impede the activation of carboxylic acids in every GlcA unit of the polysaccharide chain. To minimize this effect, the protection of some hydroxyl groups of chondroitin was planned. A benzylidene ring was installed at 4,6-diol of GalNAc units by reaction with α,α-dimethoxytoluene in the presence of (+)-camphor-10-sulfonic acid (CSA) as an acid catalyst.[29] Exhaustive protection of all GalNAc 4,6-diols of the polymer chain (DS = 1.00) was demonstrated by relative integration of the benzylidene methine proton (δ = 5.53 ppm) and acetyl (δ = 1.76 ppm) signals in the ^{1}H NMR spectrum of the protected polysaccharide **3** (see Supporting Information). This chondroitin derivative was then

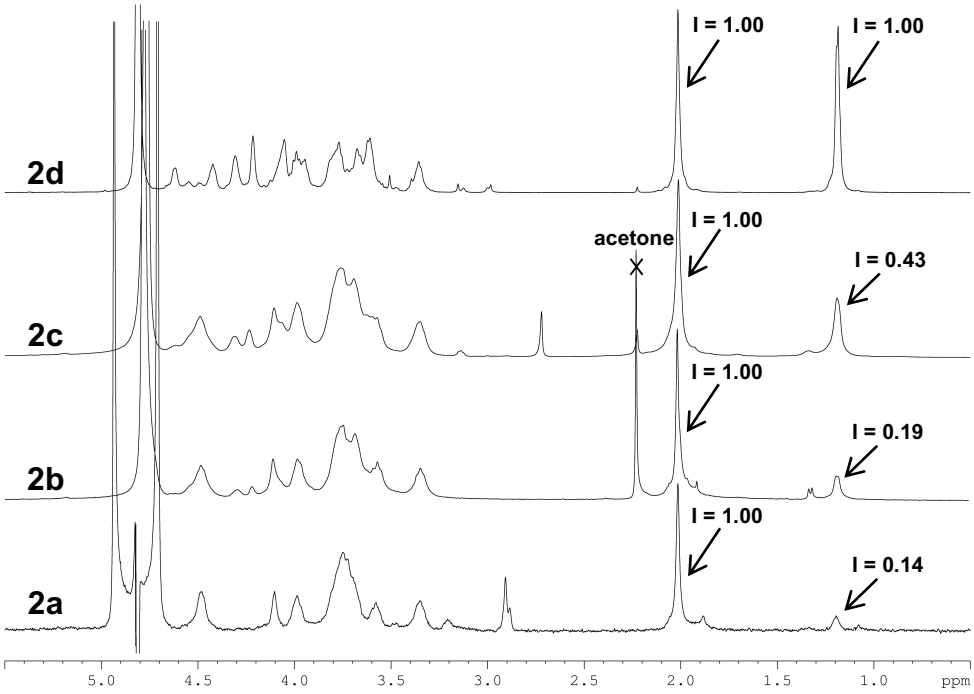


Figure 2: ^{1}H-NMR spectra (600 MHz, D_2O, 298K) of polysaccharides **2a-d** (I = Integral area)

subjected to amide coupling with **1** under the activation condition that performed best on the unprotected polysaccharide (TBTU/2,4,6-collidine). By addition of diisopropyl ether to the reaction mixture, polysaccharide **4** could be isolated by precipitation. Its ^{1}H-NMR spectrum could not give an exact DS evaluation, as methyl signal of linked Thr amide moiety (δ = 1.09 ppm) partially overlapped with the same kind of signal from free Thr amine **1** (δ = 1.02 ppm), that co-precipitated with **4** (see Supporting Information). A two step deprotection of **4** was performed by firstly treating it with $NaBrO_3$ and $Na_2S_2O_4$ in H_2O-ethyl acetate mixture to cleave oxidatively the benzylidene ring[52] and give, either at position *O*-4 or *O*-6 of GalNAc units, Bz esters,[29] that were then hydrolysed under alkaline aqueous conditions together with Thr methyl ester to afford polysaccharide **2d**. To our delight, integration of its ^{1}H-NMR spectrum showed a quantitative decoration (DS = 1.00) of GlcA with Thr amides (Figure 2). Attribution of chemical shift values of polysaccharide **2d** (Table 1) was possible by analysis of ^{13}C and two-dimensional NMR spectra (COSY, TOCSY, NOESY, HSQC-DEPT) (Figure 3 and Supporting Information) and their comparison with the data reported in the literature for unsulfated chondroitin.[53] In particular, the density at $\delta_{H/C}$ 4.21/61.0 ppm in the HSQC-DEPT spectrum could be associated to the *CH* group at position α of Thr units linked through an amide bond to GlcA carboxylic moieties.

Table 1: ^{1}H (plain) and ^{13}C NMR (italic) chemical shift attribution of **2d**[a,b]

	GlcA	GalNAc		Thr
1	4.62 *105.0*	4.42 *100.0*	α	4.21 *61.7*
2	3.35 *72.9*	3.99 *51.6*	β	4.30 *68.4*
3	3.62 *74.2*	3.80 *81.6*	γ	1.20 *19.9*
4	3.94 *77.1*	4.07 *74.5*	other signals	COOH: *176.9*
5	4.05 *68.7*	3.60 *75.6*		
6	-- *170.3*	3.67-3.76 *61.7*		
other signals	--	$NHCOCH_3$: *175.7* $NHCOCH_3$: 2.01, *23.1*		

[a] NMR experiments conducted in D_2O (600 MHz, 298 K).
[b] Chemical shifts expressed in δ relative to internal acetone [^{1}H: $(CH_3)_2CO$, δ = 2.22 ppm; ^{13}C: $(CH_3)_2CO$, δ = 30.9 ppm]

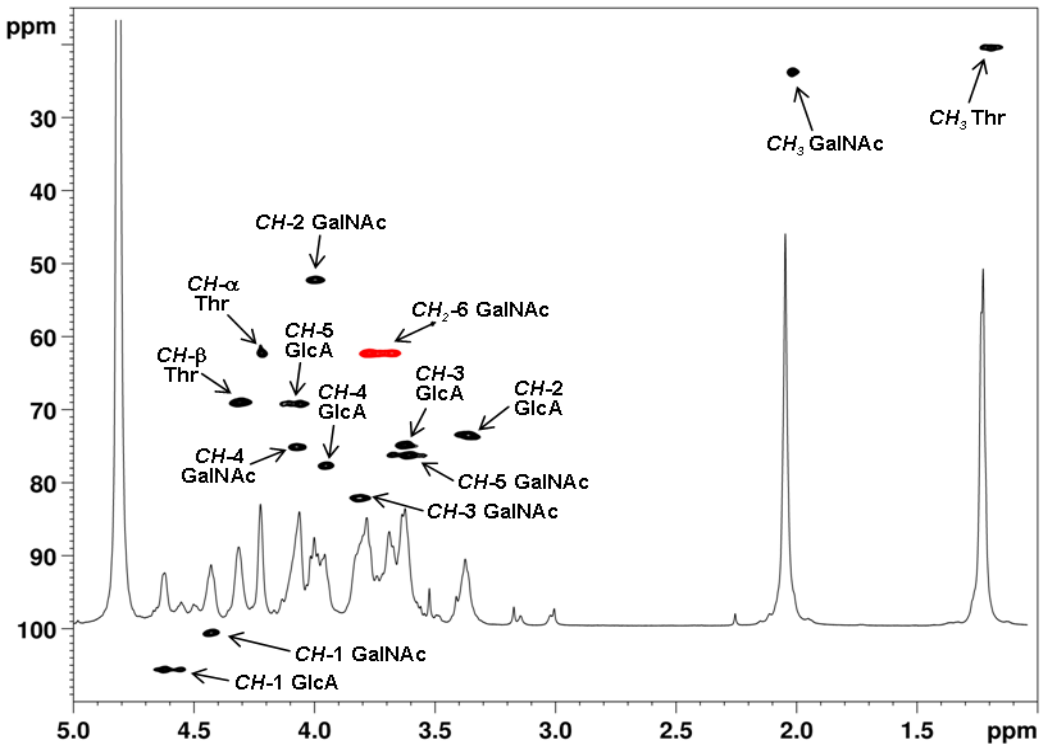


Figure 3: ^{1}H and HSQC-DEPT NMR spectra of polysaccharide **2d**

(600 MHz, D_2O, 298K; CH and CH_3 signals in black colour, CH_2 signal in red colour)

The mass-average molecular weight of the starting chondroitin and of the semi-synthetic polysaccharides **2a-2d** was determined by means of the Static Light Scattering (SLS) through the Zimm plot,[35] affording values of 41.0 ± 2.0, 37.2 ± 1.9, 35.1 ± 1.8, 22.4 ± 1.1, and 16.9 ± 0.9 Kg mol^{-1}, respectively. In Figure 4 the Zimm plot for **2c** is reported (see also Supporting Information). It is worth noting that, in spite of a molecular weight decrease observed for all the semi-synthetic

derivatives, in no case was the polysaccharide reduced from the original degree of polymerization (DP) of approx. 102 ± 5 to less than 34 ± 2, as calculated with disaccharide repeating unit molecular mass values of 401 and 502 g mol^{-1} for the starting chondroitin and the fully decorated Thr derivative **2d**, respectively. This demonstrated that the polysaccharide structure of chondroitin was conserved for derivatives **2a**-**2d**, even if a polymer chain shortening was detected in all cases. The highest shortening was observed for polysaccharide **2d**, as expected from the higher number of semi-synthetic steps required for its production with respect to **2a**-**2c** cases. In particular, the first step towards **2d** was a benzylidene ring installation on GalNAc *O*-4,6-diol (Scheme 1), requiring a high reaction temperature (80°C) and an acid catalyst such as CSA. Such conditions could break the glycosidic linkages of the chondroitin polysaccharide in a non negligible amount.

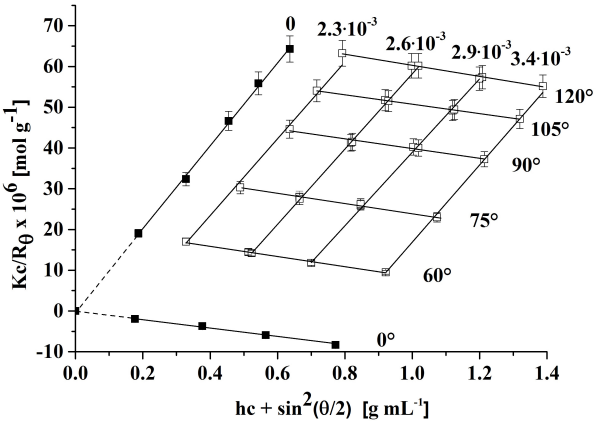


Figure 4: Zimm plot for the molecular weight determination of **2c**

With the aim of determining the three-dimensional arrangement of the fully Thr-decorated polysaccharide **2d**, an in-depth analysis of the NMR was performed. The NOE connectivities in the NOESY spectra between 1,3-diaxial protons [*H*-1/*H*-3/*H*-5 and *H*-2/*H*-4 for GlcA(Thr) and *H*-1/*H*-3/*H*-5 for GalNAc], unambiguously indicated that both sugar moieties assume the classical $^4C_1$ chair conformation. NOESY spectra also showed interesting inter-residue NOEs that were diagnostic for the determination of the spatial evolution of the polysaccharide. Particularly, strong NOEs between *H*-1 of GlcA(Thr) and *H*-3 of GalNAc, and between *H*-1 of GalNAc and *H*-4 of GlcA(Thr) indicated the relative spatial orientation of the sugars. The lack of long-range NOEs suggested that the overall structure of the polysaccharide is fairly linear. This structural information was employed to construct a simplified model of the semi-synthetic chondroitin polysaccharide [made up by six repetitions of the GlcA(Thr)-GalNAc disaccharide subunit] through NMR-restrained simulated annealing (SA) calculations. An ensemble of 400 isoenergetic structures was calculated featuring for all the considered distances a maximum violation of 0.12 Å. Subsequently, different conformations of this ensemble were clustered considering the position of the sugar ring atoms belonging to the central 4 disaccharide subunits with a root-mean-squared difference (rmsd) value of 1.5 Å. Clustering of the obtained 400 conformations resulted in 38 different conformational families among which cluster 3 represented the 25% of the total ensemble demonstrating a good

convergence of our calculations toward a well-defined structure. For this cluster, the representative conformation was considered (i.e. the one closest to the centroid of the cluster) for further 310 K molecular dynamics (MD) simulations aimed at inspecting the thermodynamic stability in explicit solvent. From these calculations (NMR-restrained SA and MD simulations), it was clear that the structure adopts a fairly linear conformation which is stable over time as demonstrated by plotting of the rmsd values during the last 10 ns of the production run. This conformation is stabilized by the formation of a series of inter-residue H-bond interactions, which are reasonably well conserved along the polysaccharide structure (Figure 5). Interestingly, a comparison between the calculated structure of **2d** and the published NMR solution structure of unsulfated chondroitin[54] (Figure 5D) reveals that the two structures are basically superimposable with no major differences in the overall conformation, thereby outlining that decoration with Thr does not induce conformational changes with respect to the unsubstituted chondroitin polysaccharide. In particular, the calculated conformation of **2d** does recall neither the "zigzag" arrangement of the *C. psychrerythraea* Thr-decorated CPS[22] nor the helical conformation very recently detected for the exopolysaccharide (EPS) secreted by the same bacteria and displaying alanine (Ala) amide groups on GalA units.[23] Interestingly, the presence of a pseudo-helical conformation in EPS allowed entrapping water molecules in discrete clefts, where no tetrahedral arrangement was detected thereby disfavouring the formation of ice crystals. From these considerations, it might be suggested that the linear structure of the semi-synthetic Thr-decorated chondroitin polysaccharide **2d** might not be conducive to ice recrystallization inhibition activity.

To confirm this, IRI activity was measured. IRI has been linked to survival in frozen environments and compounds which display IRI activity can enhance cellular cryopreservation by reducing ice growth during freezing.[55] In order to test the IRI activity, a modified 'splat' assay was used.[47] Briefly, 10 μL droplets of the polysaccharide in PBS was dropped down a tube onto a cooled (~ −70 °C) glass cover slip, and then transferred to a microscope set at −8 °C. After 30 min, the mean largest grain size (MLGS) was measured and reported relative to a PBS control. It is important to note that in this assay, MLGS values below ~ 20% indicate zero growth (as the initial crystals cannot have zero size). The IRI activity of the polysaccharides is reported in Figure 6 alongside a negative control (PEG) and another polysaccharide, dextran (M_W ~ 40 kg.mol^{-1}). Compared to PEG, the polysaccharides do have some IRI activity, reducing the grain size by up to 40 % compared to PBS alone. It is important to note that very few macromolecules have this activity, even weak, and the magnitude is compared to poly(ampholytes) which are an emerging class of macromolecular cryoprotectants.[56,57]

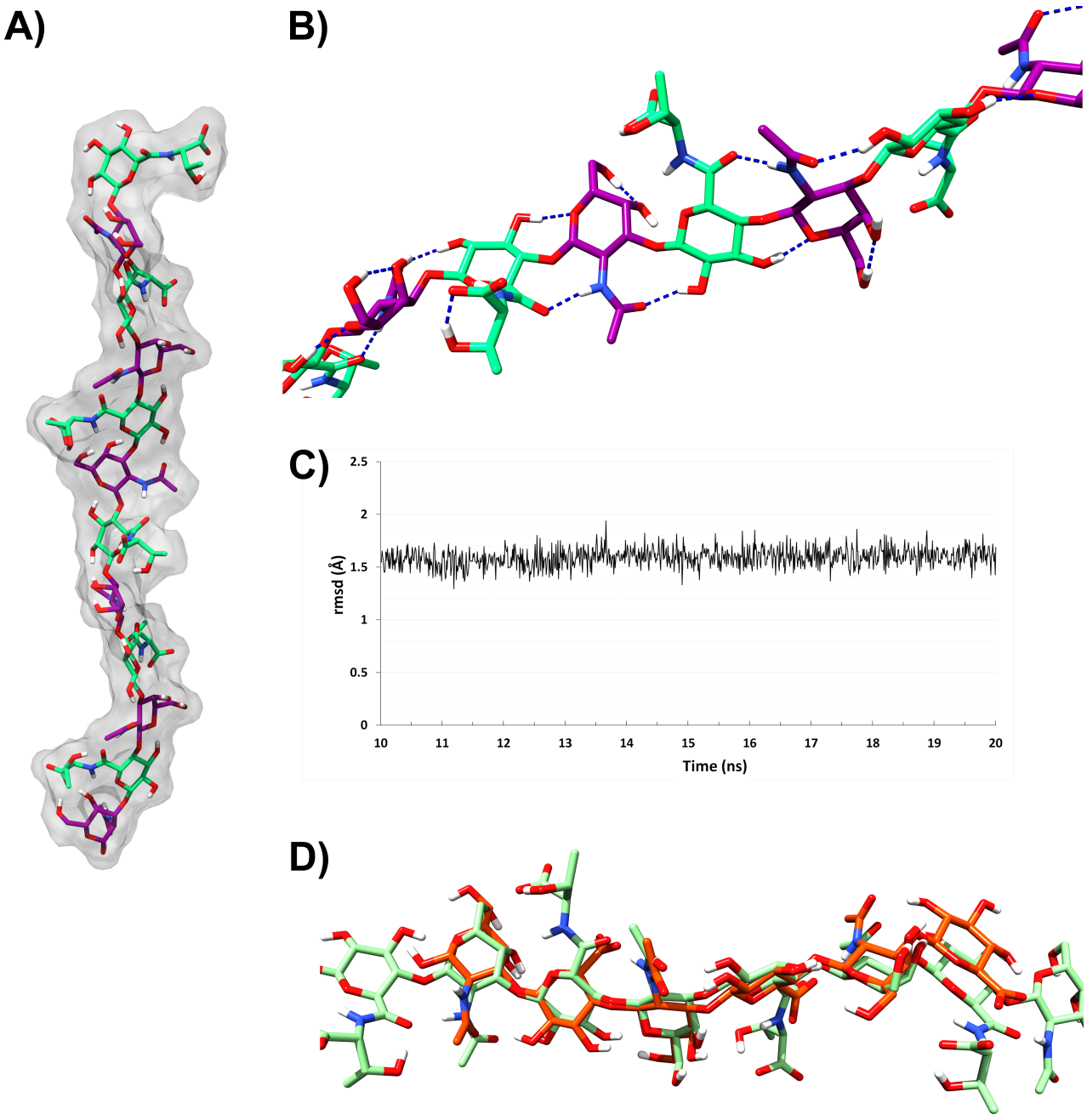


Figure 5. Front (A) and zoomed (B) view of the representative structure of the inspected polysaccharide as calculated by restrained SA calculations. GlcA(Thr) and GalNAc are represented as green and purple sticks. H-bonds are represented as dashed blue lines. C) rmsd fluctuations of the studied of the polysaccharide heavy atoms, along the last 10 ns of the MD simulation with respect to the conformation reported in (A). D) Superimposition between the structure of inspected polysaccharide **2d**, as calculated by restrained SA calculations (green sticks), and the published solution structure of unsulfated chondroitin[54] (orange sticks, PDB 2KQO).

The activity seems similar to dextran, which is only a weak ice recrystallization inhibitor. Interestingly, there is a trend that as the threonine content increases, the IRI activity is reduced and in un-modified chondroitin, the IRI activity is less

than for the threonine decorated samples. This could be associated to the slight decrease of the molecular weight measured from polysaccharide **2b** (DS_{Thr} = 0.19, M_w 35 ± 2 kg mol^{-1}) to **2c** (DS_{Thr} = 0.43, M_w 22 ± 1 kg mol^{-1}) and then **2d** (DS_{Thr} = 1.00, M_w 17 ± 1 kg mol^{-1}). Indeed, it should be noted that IRI activity has been observed in nearly all systems to increase with molecular weight.[58] Although there have been several examples of Thr- and/or Ala-rich (glyco)proteins and polysaccharides acting as antifreeze agents in Nature,[22,58,59] it is clear from these data that Thr decorations alone is insufficient for introduce potent IRI activity, or that the precise sequence/location of the threonine units in naturally-occurring complex polysaccharides might be different to these semi-synthetic analogues. Also, increasing the hydrophobicity (i.e. more threonine) alone is not enough to enhance IRI activity, in line with observations on synthetic polymers.[26,56] We did not seen any clear trends in relationship to the effect of MW, but a wider range of sampels would be required to draw further conclusions on this.

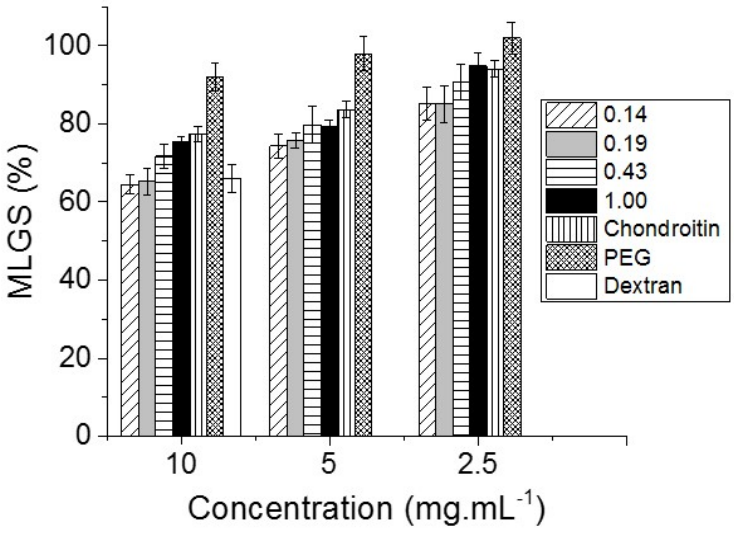


Figure 6. IRI activity of the polysaccharides **2a**, **2b**, **2c**, and **2d** (0.14, 0.19, 0.43 and 1.00 indicate their DSs of Thr amidation), chondroitin, PEG (negative control) and dextran (positive control). MLGS is expressed as a percentage of PBS buffer, and small MLGS values indicate increased IRI activity

Conclusions

In this work a microbial sourced chondroitin polysaccharide was decorated with Thr amides at its carboxylic acid moieties, in order to obtain a semi-synthetic mimic of the CPS from *C. psychrerythraea*. To this aim, several amide coupling conditions were screened in order to obtain a quantitative DS for Thr decoration. By performing the reaction on underivatized chondroitin rather low DSs (0.14-0.43) were obtained whichever the kind of carboxylic acid activator (carbodiimide-, phosphonium- or uronium-type) was employed, whereas a quantitative DS could be gained by performing the reaction on a partially protected polysaccharide derivative. This result was ascribed to a weakening of the hydrogen

bond network acting in DMF – the solvent used for the amidation reaction – after masking some hydroxyls of the polysaccharide with the protecting groups.

The three-dimensional arrangement of the Thr-decorated polysaccharide was then investigated by NOESY NMR and SA and MD simulations, determining a stable, fairly linear conformation. This was rather different from the "zigzag" arrangement determined for the CPS from *C. psychrerythraea*.[22] This difference explained the much lower IRI activity measured for the semi-synthetic polysaccharide with respect to the natural one. Indeed, the absence of discrete clefts in the linear structure of the former does not allow the entrapment of water molecules in a non tetrahedral arrangement in the first hydration shell, that disfavors the formation of ice crystals. Therefore, this work demonstrates that in spite of several examples of Thr- and/or Ala-rich (glyco)proteins and polysaccharides having some ice recrystallization inhibition activity,[22,59,60] the incorporation of more threonine, and hence increasing hydrophobicity, does not increase the magnitude of the ice recrystallization inhibition activity despite the apparent role of related polysaccharides in cryoprotection. However, it is worth noting that also the presence of a polysaccharide structure with discrete clefts such as in a pseudo-helical conformation has been recently demonstrated to be not a sufficient condition alone for IRI activity. Indeed, a very recently discovered Thr/Ala free CPS, still isolated from *C. psychrerythraea* and displaying a helical structure, does not show any IRI activity.[61] All these findings will help future investigations towards the development of (semi-) synthetic polymer mimics of natural antifreeze agents to be applied in the cryopreservation field.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Copies of 1D- and 2D-NMR spectra and Zimm plots for the molecular weight determination of the polysaccharides.

Acknowledgments

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